

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/01, A23J 3/34, A23C 9/123, 9/12	A1	(11) International Publication Number: WO 97/00078 (43) International Publication Date: 3 January 1997 (03.01.97)
(21) International Application Number: PCT/FI96/00350 (22) International Filing Date: 12 June 1996 (12.06.96) (30) Priority Data: 952926 14 June 1995 (14.06.95) FI (71) Applicant (for all designated States except US): VALIO OY [FI/FI]; Meijeritie 6, FIN-00370 Helsinki (FI). (72) Inventors; and (75) Inventors/Applicants (for US only): ISOLAURI, Erika [FI/FI]; Tallmonkuja 6, FIN-21120 Raisio (FI). METSÄNIITTY, Leena [FI/FI]; Kuusmiehentie 44 C, FIN-00670 Helsinki (FI). KORHONEN, Hannu [FI/FI]; Lemmenmäentie 28 A 3, FIN-11120 Riihimäki (FI). SALMINEN, Seppo [FI/FI]; Puutarhakatu 2 C 32, FIN-20100 Turku (FI). SYVÄOJA, Eeva-Liisa [FI/FI]; Tonttutyönkuja 3 D 21, FIN-02200 Espoo (FI). (74) Agent: OY JALO ANT-WUORINEN AB; Iso Roobertinkatu 4-6 A, FIN-00120 Helsinki (FI).		(81) Designated States: AU, BG, BR, CA, CN, CZ, EE, IL, JP, KE, KP, KR, LT, LV, MX, NO, NZ, PL, RO, RU, SG, SK, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: METHODS OF PREVENTING OR TREATING ALLERGIES		
(57) Abstract The present invention relates to methods and means of suppressing food-induced hypersensitivity reactions in patients suffering from food allergy. Particularly the invention provides methods of making a protein hydrolysate formula for downregulating hypersensitivity reactions and for promoting gut immune barrier, and methods of preventing or treating allergies, especially cow's milk allergy in infants by fortifying the cow's milk elimination diet with suitable probiotic bacteria derived from gastrointestinal tract, especially Lactobacilli. In addition, administering fragments of an allergen modified by gastrointestinal bacteria immune response can be "turned around" from sensitization to systemic hyporesponsiveness. A preferable bacterium to be used in this invention is <i>Lactobacillus</i> GG (ATCC 53103).		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

Methods of preventing or treating allergies

Field of the invention

5 The present invention relates to methods and means of suppressing food-induced hypersensitivity reactions in patients suffering from food allergy. Particularly the invention provides methods of preventing or treating allergies, especially cow's milk allergy in infants. The
10 invention also relates to development of specific formulae for allergic infants with impaired gut barrier function.

Cow's milk allergy (CMA) is defined as an immune-mediated
15 adverse reaction to cow's milk proteins. The present treatment of choice is the complete elimination of cow's milk antigens. In infants with CMA, it is necessary to use a substitute formula when human milk is unavailable. Hydrolysed formulae, based on cow's milk-derived whey or
20 casein, are used to provide adequate nutrition with a reduced antigenic load. The preliminary heat treatment of cow's milk mainly affects the conformation of proteins and facilitates their hydrolysis. Subsequent enzymatic hydrolysis with pepsin, trypsin, pancreatic extracts and
25 extracts from the intestinal mucosa causes progressive destruction of sequential epitopes and refines the formulae into the least antigenic and allergenic form.

In most cases, extensively hydrolysed cow's milk-derived
30 formulae can be safely introduced and these are efficient and clinically and metabolically well tolerated. Enzymatic hydrolysis, however, does not necessarily make the formula nonallergenic, as the optimal extent of hydrolysis is not known and traces of the original protein are
35 detected in the hydrolysate. Therefore, introduction of these substitutes to children with cow's milk allergy must be cautious.

The approach to control allergic inflammation by antigen elimination has not been satisfactory, particularly in patients with multiple food allergies (Sampson et al., 1992). These patients frequently show increased intestinal permeability and dysfunction of the intestine's defence barrier (Majamaa et al., 1996, Majamaa and Isolauri, 1996). This enhances the risk for growth disorders and sensitization to multiple foods. New approaches are urgently needed for the treatment of cow's milk allergy to improve the substitute formulae in CMA.

Intestinal antigen handling determines subsequent immune response to the antigen. In health, antigens are absorbed across epithelium along two functional pathways. The main pathway is degradative reducing the immunogenicity of the antigen. A minor pathway allows the transport of intact proteins which is crucial for antigen-specific immune responses. Aberrant antigen absorption enhances the sensitization process (Fargeas et al., 1995).

Differential production of cytokines by T-helper (Th) cells during an immune reaction has important regulatory effects on the nature of the immune response. The cytokine profile of the natural immune response determines the phenotype of the subsequent specific immune response. Apart from controlling IgE synthesis, IL-4 is crucial for the development and maturation of the Th2 phenotype, characterized for allergic inflammation.

This process appears crucial for the development of tolerance to ingested protein. Oral tolerance is a state of antigen-specific systemic non-responsiveness characterized by local antigen-specific IgA response.

An isolated human intestinal strain, *Lactobacillus* strain GG (*Lactobacillus* GG, ATCC 53103) has recently been shown to promote local IgA responses against dietary antigens

encountered by the enteric route and may therefore aid in immune elimination (Isolauri *et al.*, 1993). It is not as yet known whether particular strains of intestinal bacteria could directly modify the immunogenicity of the food antigens and consequently downregulate hypersensitivity reactions.

Lactobacilli are included in the microbial flora of healthy intestines. It has been assumed that Lactobacilli act in the intestinal tract by competing for receptors and nutrients against pathogenic microbes on the intestinal mucosa.

Probiotics are viable microbial preparations which promote health by maintaining the natural microflora in the gut. A microbial preparation can be acknowledged as a probiotic if the functioning microbes thereof and their mode of action are known. The probiotics attach on the intestinal mucosa, colonize the human intestinal tract and prevent attachment of harmful microbes. A crucial presumption is that they get up to the gut's mucosa and do not get destroyed in the upper part of the gastrointestinal tract. *Lactobacillus* GG is one of known bacteria having probiotic characteristics.

25

Description of the invention

The present inventors have now found that certain bacteria of the gastrointestinal tract, especially Lactobacilli, and especially bacteria having probiotic characteristics, can be used to enhance the efficacy of elimination diets and to improve the oral tolerance, in preventing or treating food-induced hypersensitivity reactions in a patient.

35

One aspect of the invention is that protein hydrolysates obtained by hydrolysis of proteins with above mentioned

gastrointestinal bacteria can also be used to said purpose. We show here that protein hydrolysates obtained according to this invention have an immunological effect promoting hypoallergenicity. The hydrolysates downregulate hypersensitivity reactions thus promoting the gut immune barrier function, i.e. stabilising the gut mucosal barrier.

The present invention provides an improved protein hydrolysate formula downregulating hypersensitivity reactions and promoting the gut immune barrier function. The hydrolysate formula is obtainable by hydrolysing proteins with enzymes derived from probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103), and with trypsin and/or pepsin.

Alternatively, a protein hydrolysate formula of the invention can be obtained by hydrolysing proteins with trypsin and/or pepsin, and adding to the hydrolysate so obtained a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103). The bacteria are added into the hydrolysate formula preferably as a lyophilized preparation.

While administering such a hydrolysate formula to a patient, it is to be expected that the hydrolysing enzymes of the bacteria present are released *in vivo* whereby the same effect is achieved as with an improved hydrolysate formula as defined above. In addition, the viable bacteria stabilize the gut mucosal barrier enhancing the local defence.

An embodiment of this invention is a method of preventing or treating food-induced hypersensitivity reactions in an infant, which method comprises the step of administering to an infant at risk the improved protein hydrolysate formula of the invention, or alternatively a protein hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

A further embodiment of this invention is a method of treating cow's milk allergy in a patient, comprising administering to the patient the improved protein hydrolysate formula of the invention or, alternatively administering a protein hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

The invention also provides a method of promoting tolerogenic immune responses to food antigens in a patient, comprising oral administration of the improved protein hydrolysate formula of the invention to the patient, or, alternatively, a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103), or, alternatively, a protein hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

In the methods of the invention as defined above, when a protein hydrolysate formula is used in combination with selected probiotic bacteria as defined above, such a formula can be any suitable protein hydrolysate formula, known or novel. It can thus be either a partial protein hydrolysate, or alternatively an extensively hydrolysed formula. Preparation of a protein hydrolysate suitable for this purpose is disclosed e.g. in EP patent application No. 601 802.

Preferred bacterium to be used in the formulae and methods of the invention is *Lactobacillus* GG (ATCC 53103).

In this specification and in the appended claims the phrase "bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103)" should be understood to define bacteria whose adhesive and colonizing characteristics are comparable to those of the LGG strain, e.g. as defined in EP-patent 199 535. These bacteria are also supposed to have same kind of enzyme system as LGG, which means that enzymes derived from such bacteria are able to degrade proteins to produce hydrolysatation products with same effects as those obtained using enzymes derived from LGG.

Abbreviations

30	CI	confidence interval
	IFN- γ	Interferon- γ
	IgA	Immunoglobulin A
	IgE	Immunoglobulin E
	IL-4	Interleukin-4
35	LGG	<i>Lactobacillus</i> GG (ATCC 53103)
	OKT3	anti-CD3 antibody
	PBMC	peripheral blood mononuclear cells

TNF- α tumour necrosis factor α
 WF test group receiving extensively hydrolysed Whey
 Formula
 WF-GG test group receiving extensively hydrolysed Whey
 5 Formula and LGG preparation

P/T-casein = casein hydrolysed by pepsin and trypsin

P/T- α_{s1} -casein = α_{s1} -casein hydrolysed by pepsin and
 trypsin

10

Brief description of the drawings

Fig. 1A-1D Mitogen-induced proliferative responses
 of PBMCs *in vitro* to P/T-casein (1A), P/T- α_{s1} -casein (1B),
 15 P/T-casein additionally hydrolysed with LGG derived enzy-
 mes (1C) and P/T- α_{s1} -casein additionally hydrolysed with
 LGG derived enzymes (1D). Results are expressed as mean
 counts per minute for cultures without hydrolysed product
 (PHA-RPMI 1640) and with hydrolysed product at three con-
 20 centrations (0.1, 10 and 1000 μ g/ml). Horizontal lines
 represent the geometric mean of counts per minute of the
 six experiments, enumerated the same for each individual.

Fig. 2 The clinical score of atopic dermatitis (SCORAD)
 25 in each patient and the median score for extent (A), in-
 tensity (B) and subjective score (C) for atopic dermati-
 tis before management (0) and one month later (I) in
 infants receiving extensively hydrolysed whey formula
 without (WF) or the same with *Lactobacillus* GG (WF-GG).

30

Fig. 3 The effect of casein and *Lactobacillus* GG-degra-
 ded casein on the production of IL-4 and IFN- γ by PBMC in
 atopic patients (a,b) and in nonatopic healthy children
 (c,d). White columns represent the median cytokine pro-
 35 duction in control cultures; black columns, in cultures
 containing purified casein; and hatched columns, in cul-
 tures containing *Lactobacillus* GG-degraded casein. Inter-

secting lines represent the upper and lower quartiles.

*Statistically significant pairwise comparison to control cultures.

- 5 **Fig. 4** The effect of α_{s1} -casein and *Lactobacillus* GG-degraded α_{s1} -casein on the production of IL-4 and IFN- γ by PBMC in atopic patients (a,b) and in nonatopic healthy children (c,d). White columns represent the median cytokine production in control cultures; black columns, in
10 cultures containing α_{s1} -casein; and hatched columns, in cultures containing *Lactobacillus* GG-degraded α_{s1} -casein. Intersecting lines represent the upper and lower quartiles. *Statistically significant pairwise comparison to control cultures.

15

The following examples illustrate the invention further. Methods for preparing the improved hydrolysate are described, as well as experiments showing the beneficial therapeutic effect observed in the present studies.

20

Experimental

Example 1 Suppression of lymphocyte proliferation *in vitro* by bovine caseins hydrolysed with *Lactobacillus* GG derived enzymes

1.a. Hydrolysis of Cow's Milk Proteins

Bovine total casein and casein components (α_{s1} -casein) were purified from cow milk (Syväoja, 1992). The hydrolysis with the enzyme mixture derived from *Lactobacillus* GG was applied separately to casein and α_{s1} -casein. The enzymes were isolated using the modified method of Exterkate and de Veer, 1985. Briefly, the enzymes were released by sonication of frozen bacterial cells. The supernatant of the centrifuged cells was separated and used for the hydrolysis of casein and α_{s1} -casein. The hydrolysis was carried out for 24 h at 34°C.

The GG-hydrolysates so obtained were further hydrolysed with pepsin and trypsin. Briefly, the samples were hydrolysed first with 0.1 % pepsin in 0.1 mol/l HCl for 3 hours at 37°C, pH 2.5. After pH adjustment with 250 mg NaHCO₃ and 2 mol/l NaOH, 0.1 % trypsin was added to the samples and these were hydrolysed for 5 hours at 37°C, pH 8.0.

P/T-casein and P/T- α_{s1} -casein samples were obtained by hydrolysing the purified casein and α_{s1} -casein only with pepsin and trypsin as described above, without GG-hydrolysatation.

1.b. Lymphocyte transformation test

A modification of the whole blood micromethod for mitogen-induced lymphocyte transformation was used. Six to eight healthy individuals volunteered as blood donors for the experiment. Briefly, heparinized venous blood was obtained and diluted 1:7 with RPMI 1640 culture medium

(Grand Island Biological Co. NY, USA) containing antibiotics. Phytohemagglutinin (PHA) (Difco Laboratories, Detroit, Mich., USA) was diluted with RPMI 1640 containing antibiotics, with ranges of final concentrations in
5 cultures from 5 to 1250 µg/ml. Lyophilized hydrolysates of casein and α_{s1} -casein were diluted in RPMI 1640 to their final concentrations of 0.1 µg/ml (low), 10 µg/ml (medium) and 1000 µg/ml (high) as proven to be nontoxic on T cells in dye exclusion studies with eosin.

10

Two sets of experiments were undertaken to investigate the mitogen-induced proliferative responses of peripheral blood mononuclear cells (PBMCs) to (A) casein hydrolysed with pepsin and trypsin (=P/T-casein), (B) α_{s1} -casein
15 hydrolysed with pepsin and trypsin (=P/T- α_{s1} -casein), (C) P/T-casein additionally hydrolysed with *Lactobacillus* GG derived enzymes, and (D) P/T- α_{s1} -casein additionally hydrolysed with *Lactobacillus* GG derived enzymes.

20 Experiment consisted of four control cultures with different dilutions of the culture medium and the mitogen, as well as corresponding test cultures with 25 µl of hydrolysed products at three different concentrations. The assay was carried out as described in Sütas et al., 1996.

25

The mitogen-induced proliferation of PBMCs was expressed as counts per minute, background excluded. The results were presented for control cultures containing 125 µg/ml PHA and RPMI 1640 and for the three test cultures con-
30 taining 125 µg/ml PHA and hydrolysed products at the low, medium, and high concentrations.

1.c. Statistical analysis

A nonparametric pairwise test (Wilcoxon signed-rank test)
35 was used to compare the change of the values in counts per minute of each of the test cultures with those of the control cultures. The level of significance was $p < 0.05$.

Significant results of pairwise comparisons were presented as suppression or stimulation according to decrease or increase of the values in counts per minute in the test culture.

5

1.d. Results

In the experiments done with P/T-casein and P/T- α_{s1} -casein additionally hydrolysed with *Lactobacillus* GG derived enzymes, the mitogen induced proliferation of PBMCs was significantly suppressed at 0.1, 10 and 1000 $\mu\text{g/ml}$ (p=0.03, p=0.03, and p=0.03) (Figs 1C and 1D) compared to P/T-casein and P/T- α_{s1} -casein (Figs 1A and 1B).

15 **Example 2. The treatment of infants with atopic dermatitis with protein hydrolysate formula supplemented with lactic acid bacteria**

2.a. Patients and study design

20 The study comprised 31 infants, aged 2.5 to 15.7 (mean age 8) months, fulfilling the Hanifin criteria (Hanifin, 1987) for atopic eczema in children. They had been referred to a paediatric clinic on the basis of atopic eczema and suspected cow's milk allergy. The mean age at onset of symptoms was 2.4 months. Duration of total breast-feeding was 5.9 months. A positive family history of atopic diseases (asthma, atopic eczema and allergic rhinitis) or food allergy in first-degree relatives was noted in 26 (84 %) patients. The eczematous lesions were treated with emollients and topical corticosteroids. No patient was receiving systemic corticosteroid therapy. Besides atopic eczema, gastrointestinal disturbances such as loose stools, vomiting or diarrhea were seen in 9 (19 %) patients. After the study periods the patients were allocated to double-blind placebo-controlled cow's milk challenge. Only those having a positive reaction (27/31) were included in the final study population.

The patients were randomized to two groups for one month. One group (WF, n=14) received extensively hydrolysed whey formula (Valio Ltd, Helsinki, Finland, EP-A-601802) and another group (WF-GG, n=13) received the same formula with additional *Lactobacillus* GG preparation (5×10^8 cfu/g) (supplied by Valio Ltd, Helsinki, Finland). After the one-month therapy with their assigned formulae; both groups received an extensively hydrolysed whey formula (Valio Ltd) for one additional month. The serum total IgE concentration, cow's milk-specific IgE (RAST, Pharmacia, Uppsala, Sweden) and skin prick tests were determined from all patients before dietary intervention. Prick testing was done on the volar aspect of the forearm, using a commercially available cow milk allergen ALK (Allergologisk Laboratorium, Horsholm, Denmark) and test formula diluted to normal feed concentration. A 1 mm single-peak lancet with shoulder to prevent deeper penetration (ALK) was used, with histamine dihydrochloride 10 mg per milliliter (ALK) as positive control. Reactions were read after 15 minutes, and a response half of the histamine reaction size or more was recorded as positive on condition that the mean diameter of the wheal was at least 3 mm and the negative control at the same time 0 mm.

Faecal samples for the determination of α -1 antitrypsin and TNF- α were collected before the commencement of the management and 1 (corresponding to the study period) and 2 months later.

The severity of atopic dermatitis was scored according to the SCORAD method, established by the European Task Force on Atopic Dermatitis (1993). Briefly, the extent (score A) of the dermatitis was estimated using the rule of nines. The intensity (score B) of the dermatitis was the sum of the individual scores (0-3) for erythema, oedema and/or papules, excoriation, lichenification and dryness. The subjective (score C) manifestations (Scored 1-10),

including pruritus and sleep loss, were assessed from parents' estimations. SCORAD was obtained with the calculation: $A/5+3.5 \times B+C$.

- 5 The study protocol was approved by the Ethical Review Committee of Tampere University Hospital. An informed consent was obtained from the parents.

2.b. Determination of α -1 antitrypsin in faecal
10 specimens

- Frozen faecal specimens were thawed at room temperature and homogenized. Approximately 1 g was transferred to a glass tube and lyophilized. The resulting dry material was ground and 50 mg transferred to an Eppendorf tube.
15 One ml of 0.15 M NaCl solution was added, and α -1 antitrypsin was extracted by vigorous mixing in a Vortex mixer for 20 minutes at room temperature. The resulting suspension was centrifuged at 25,000 g for 10 minutes to remove the debris, and the supernatant was used for the
20 determination of α -1 antitrypsin using a Behring BNA nephelometer according to the manufacturer's instructions. The results are given as mg/g dry weight of lyophilized faeces.

- 25 2.c. Determination of TNF- α in faecal specimens
Deep-frozen faecal specimens were thawed at room temperature, suspended 1:1 (w/v) in physiological saline and allowed to sediment. Of the supernatant, 0.5-1.0 ml was transferred to an Eppendorf tube and centrifuged at
30 25,000 g for 10 minutes. The supernatant was then used for the determination of TNF- α . A commercial enzyme immunoassay (Human TNF- α ELISA Kit, Endogen Inc., Boston, Massachusetts, USA) was used, as instructed for serum specimens, for the determinations of faecal TNF- α .

2.d. Statistics

By reason of the skewed distributions of serum IgE concentrations, logarithmic (ln) transformation was used and data are presented as means with 95 % confidence intervals (CI). The concentrations of inflammatory parameters are presented with medians with lower and upper quartiles. The Wilcoxon signed rank test and the Mann-Whitney U-test were used in statistical comparisons.

10 2.e. Results

2.e.1. Clinical data

The mean (95 % CI) serum total IgE was 31 (15-61) kU/l in patients receiving the hydrolysate formula. RAST for cow's milk was positive (>0.4 kU/l) in 10/31 (37 %) atopic eczema patients. Skin prick test for cow's milk was positive in 8/31 (30 %) patients.

The severity of atopic dermatitis in each patient before the commencement of the managements and one month later, i.e. after the study period are presented in Figure 2. The median (lower quartile-upper quartile) SCORAD score was 21(14-31) in group WF and 26 (17-38) in group WF-GG before management ($p=0.33$). There was a significant improvement of SCORAD score after one month's intervention in those receiving *Lactobacillus* GG ($p=0.008$), but not in those receiving extensively hydrolysed formula without *Lactobacillus* GG ($p=0.89$). The SCORAD score was then 19 (13-31) in group WF and 15 (7-28) in group WF-GG. The decrease in the SCORAD score in WF-GG was due to the reduction of the extent (score A, $p=0.004$), intensity (score B, $p=0.05$) and subjective score (score C, $p=0.01$) for atopic dermatitis (Figure 2). The improvement of SCORAD score was achieved by 2 months in the WF group, and in group WF-GG in remained unchanged after cessation of *Lactobacillus* GG. At 2 months, the median (lower quartile-upper quartile) SCORAD score in group WF was 14(2-38) and in group WF-GG 16(6-25).

2.e.2. The concentrations of α -1 antitrypsin and TNF- α in faeces

In healthy controls (n=9), the median (lower quartile-upper quartile) concentration of α -1 antitrypsin was 0.5 (0.5-1.7) mg/g. The concentration of α -1 antitrypsin was comparable between groups WF and WF-GG before management (p=0.22). As indicated in Table 1, the concentration of α -1 antitrypsin decreased significantly in group WF-GG (p=0.03), but not in group WF (p=0.68) during the one month study period. At two months, the concentration of α -1 antitrypsin was 1.2 (0.5-1.6) in WF and 0.5 (0.5-0.7) in WF-GG.

The concentration of faecal TNF- α was 0 (0-0.08) pg/g in healthy controls. The concentration of faecal TNF- α was significantly higher in atopic children, p<0.0001 (Table 1). The concentration of TNF- α was comparable between the groups WF and WF-GG before management (p=0.57). The concentration of faecal TNF- α decreased significantly in WF-GG (p=0.003) but not in WF (p=0.38) during the one month study period (Table 1). A reduction in TNF- α concentration was achieved by 2 months in group WF, while in group WF-GG who were also given the extensively hydrolysed formula without *Lactobacillus* GG a trend to increased TNF- α was detected. The concentration of TNF- α was then 84 (25-129) in WF and 144 (20-338) in WF-GG.

Table 1. The concentrations of faecal α -1 antitrypsin and TNF- α before management (0) and one month later (I) in infants receiving extensively hydrolysed whey formula (WF) or the same formula containing *Lactobacillus* GG bacteria (WF-GG). Data denote median (lower quartile-upper quartile).

	WF	WF-GG
α -1 antitrypsin 0 (mg/g)	1.7 (1.5-2.3)	1.4 (0.5-1.9)
α -1 antitrypsin I (mg/g)	1.7 (1.1-2.8)	0.5 (0.5-1.0)
TNF- α 0 (pg/g)	632 (126-1880)	709 (91-1131)
TNF- α I (pg/g)	494 (147-1009)	34 (19-103)

Example 3. Downregulation of cytokine production by peripheral blood mononuclear cells in atopic children

3.a. Patients and methods

Altogether, 14 patients aged 5-29 (mean, 16) months who fulfilled the Hanifin criteria for atopic dermatitis (Hanifin, 1987) and eight age-matched non-atopic healthy children were studied. None were receiving systemic corticosteroids at the time of the study.

OKT3 (anti-CD3-antibody) containing ascites fluid was a kind gift from Dr M. Kaartinen, Department of Bacteriology and Immunology, University of Helsinki, Helsinki, Finland. Bovine total casein had been purified from bovine milk as described in Syväoja (1992). Purified casein was hydrolysed with *Lactobacillus* GG-derived enzymes as described in Example 1. Purified casein or *Lactobacillus* GG-degraded casein were lyophilized and stored at room temperature. Before experiments, they were diluted in

RPMI 1640 and filter sterilization (0.1 μ m, Millipore corporation, Bedford, MA, USA) was applied.

Complete culture medium consisted of RPMI 1640 with 10 %
5 foetal calf serum, 10 mM Hepes buffer, 2 mM L-glutamin
(Gibco Life Technologies, Paisley, UK), 50 U/ml benzyl-
penicillin (Sigma, St. Louis, MO, USA), 10 mg/ml gentamy-
cin (Roussel Laboratories Ltd, Uxbridge, Middlesex, UK).
Peripheral venous blood (5 ml) was obtained. PBMC con-
10 taining 90 % lymphoid cells was isolated by FicollPaque
(Pharmacia Biotech, Uppsala, Sweden) gradient centrifuga-
tion and suspended at 1×10^6 cells/ml in complete culture
medium. Culture wells were precoated with anti-CD3 anti-
body containing ascites fluid at a pretested optimal di-
15 lution. The test culture additionally contained dilution
of casein or *Lactobacillus* GG-degraded casein at a final
concentration of 1 mg/ml. These experiments were repeated
for purified bovine α_{s1} -casein or *Lactobacillus* GG-degra-
ded α_{s1} -casein. After 24 h of incubation in a humidified 5
20 % CO₂ atmosphere at 37°C, supernatants were collected and
stored at -70°C for cytokine assays. IL-4 and IFN- γ in
culture supernatants were determined by commercially
available ELISA kits according to manufacturers' instruc-
tions (IL-4: CLB, Compact Human Interleukin-4 ELISA kit,
25 Central Laboratory of The Netherlands Red Cross Blood
Transfusion Service, Amsterdam, The Netherlands; IFN- γ .
EIFNG, Endogen Inc., Cambridge, MA, USA). The results
from different runs were equalized employing the compa-
rison of standard curves and were expressed as pg/ml. The
30 sensitivity of the assays for IL-4 was 2.3 pg/ml and for
IFN- γ , 5 pg/ml. Wilcoxon signed-rank test was used in
statistical comparisons of the test cultures to the cont-
rol cultures. The level of significance was $P < 0.05$.

3. b. Results

In atopic patients, both IL-4 and IFN γ production were increased in cultures containing purified casein when compared to control cultures, $P = 0.008$ and $P = 0.008$,
5 respectively (Fig. 3a,b). No such effect of bovine casein was observed when degraded by enzymes of *Lactobacillus* GG. Conversely, the IL-4 production in cultures containing *Lactobacillus* GG-degraded casein was significantly less than in control cultures, $P = 0.003$ (Fig. 3a),
10 and the IFN- γ production in these cultures was comparable to control cultures, $P = 0.10$ (Fig. 3b).

In healthy children, the production of IL-4 and IFN- γ in cultures containing purified casein were comparable to
15 control cultures, $P = 0.10$ and $P = 0.10$ (Fig. 3c,d). In parallel to the findings in atopic patients, healthy children had significantly less IL-4 production in cultures containing *Lactobacillus* GG-degraded casein than in control cultures, $P = 0.01$ (Fig. 3c) and the IFN- γ production in these cultures remained comparable to control
20 cultures, $P = 0.50$ (Fig. 3d). Parallel results were obtained with α_{s1} -casein and *Lactobacillus* GG-degraded α_{s1} -casein (Fig. 4).

References

- 5 European task force on atopic dermatitis. Severity scoring of atopic dermatitis: the SCORAD index. *Dermatology* 1993; 186:23-31.
- 10 Exterkate F and de Veer G. Partial isolation and degradation of caseins by cell wall proteinase(s) of *Streptococcus cremoris*. *Appl Environ Microbiol* 1985; 49:328-32.
- 15 Fargeas MJ, Theodorou V, More J, Wal JM, Fioramonti J, Bueno L. Boosted systemic immune and local responsiveness after intestinal inflammation in orally sensitized guinea pigs. *Gastroenterology* 1995; 109:53-62.
- Hanifin JM. Epidemiology of atopic dermatitis. *Monogr Allergy* 1987; 21:116-131
- 20 Isolauri E, Majamaa H, Arvola T, Rantala I, Virtanen E, Arvilommi H. *Lactobacillus casei* strain GG reverses increased intestinal permeability induced by cow milk in suckling rats. *Gastroenterology* 1993; 105:1643-1650.
- 25 Majamaa H, Isolauri E. Evaluation of gut mucosal barrier: evidence for increased antigen transfer in children with atopic eczema. *J Allergy Clin Immunol* 1996, in press.
- 30 Majamaa H, Miettinen A, Laine S, Isolauri E. Intestinal inflammation in children with atopic eczema: faecal eosinophil cationic protein and tumor necrosis factor- α as noninvasive indicators of food allergy. *Clin Exp Allergy* 1996; 26:181-187.
- 35 Sampson HA, James MJ and Bernhisel-Broadbent J. Safety of an amino acid-derived infant formula in children allergic to cow milk. *Pediatrics* 1992; 90:463-465.
- 40 Sütas Y, Soppi E, Korhonen H, Syväoja EL, Saxelin M, Rokka T, Isolauri E. Suppression of lymphocyte proliferation *in vitro* by bovine caseins hydrolyzed with *Lactobacillus casei* GG derived enzymes. *J Allergy Clin Immunol* 1996, in press.
- 45 Syväoja EL. Quantitative determination of the main casein components and purification of α_{s2} - and κ -casein from bovine milk. *Milchwissenschaft* 1992; 47:563-566

Claims

1. A method of making a protein hydrolysate formula for downregulating hypersensitivity reactions and for promoting gut immune barrier, comprising the steps of
5 hydrolysing proteins with enzymes derived from a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103), and with pepsin and/or trypsin.
10
- 2.. The method as defined in claim 1 wherein the enzymes are derived from a bacterial preparation comprising
15 *Lactobacillus* GG (ATCC 53103).
3. A method of making a protein hydrolysate formula for downregulating hypersensitivity reactions and for promoting gut immune barrier, comprising the steps of
20 hydrolysing proteins with pepsin and/or trypsin, and adding to the hydrolysate a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).
25
4. The method as defined in claim 3 wherein the bacterial preparation comprises *Lactobacillus* GG (ATCC 53103).
- 30 5. A protein hydrolysate formula for downregulating hypersensitivity reactions and for promoting gut immune barrier, which hydrolysate is obtainable by the method as defined in any one of claims 1 to 4.
- 35 6. A method of preventing or treating food-induced hypersensitivity reactions in an infant, comprising

administering to an infant at risk the protein hydrolysate formula according to claim 5.

5 7. A method of treating cow's milk allergy in a patient, comprising administering to the patient the protein hydrolysate formula made according to claim 1.

10 8. A method of promoting tolerogenic immune responses to food antigens in a patient, comprising oral administration of the protein hydrolysate formula according to claim 5.

15 9. A method of preventing or treating food-induced hypersensitivity reactions in an infant, comprising administering to an infant at risk a protein hydrolysate formula made according to claim 1 together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

25 10. A method of treating cow's milk allergy in a patient, comprising administering to the patient a protein hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

30

11. A method of promoting tolerogenic immune responses to food antigens in a patient, comprising oral administration of a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103), or a protein

35

hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially *Lactobacilli*, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

12. The method according to any one of claims 9 to 11 wherein the bacterial preparation comprises *Lactobacillus* GG (ATCC 53103).

1/5

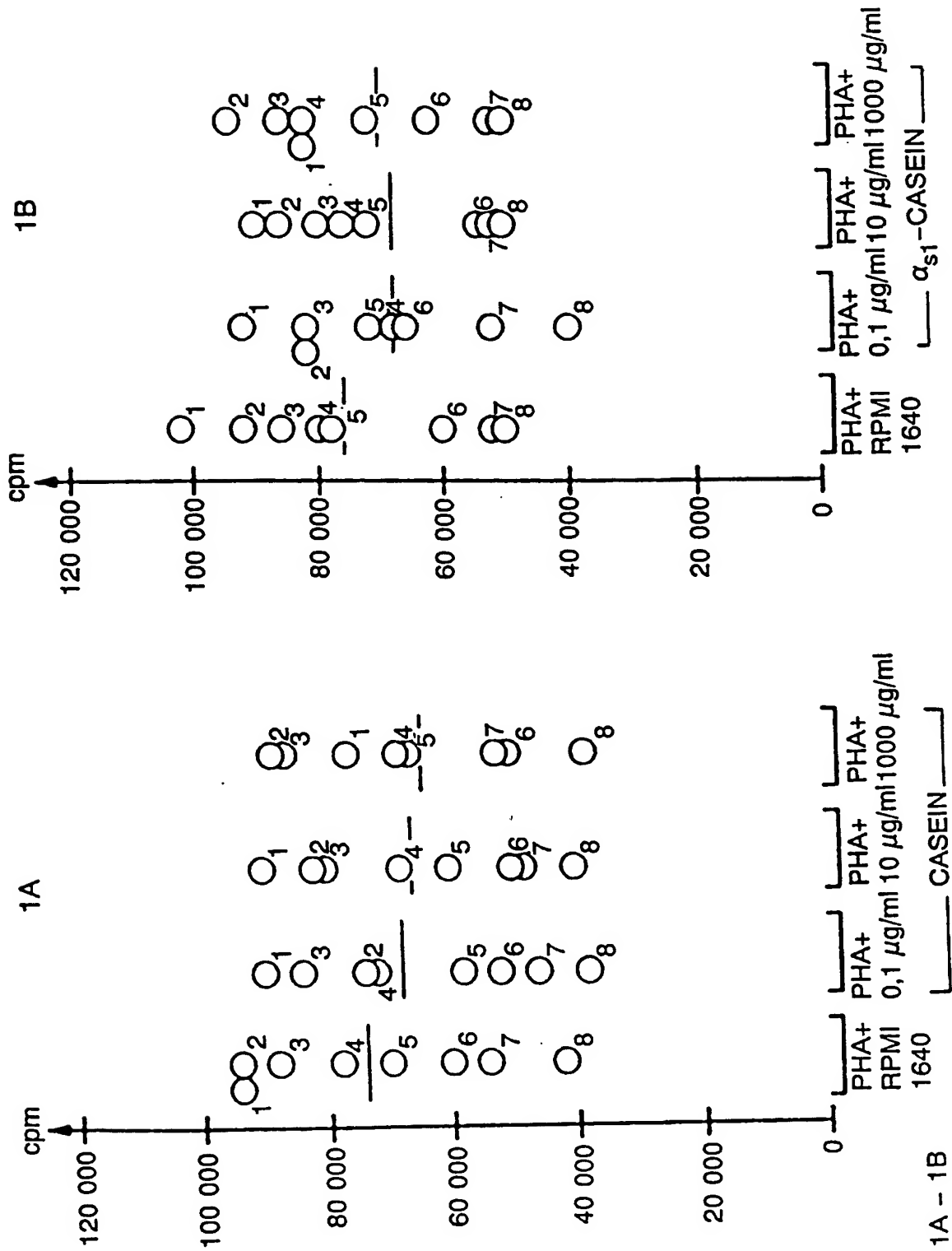


Figure 1A - 1B

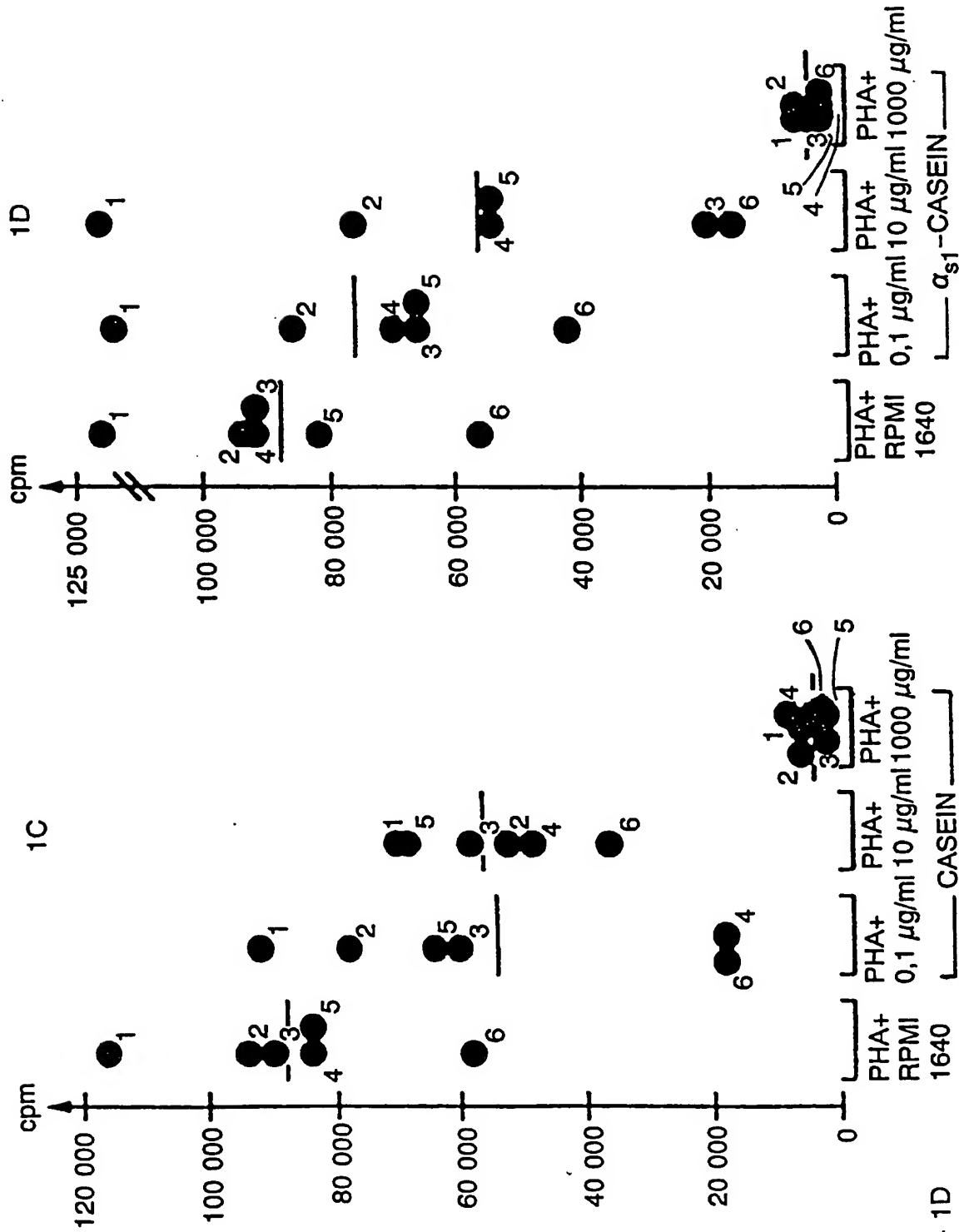
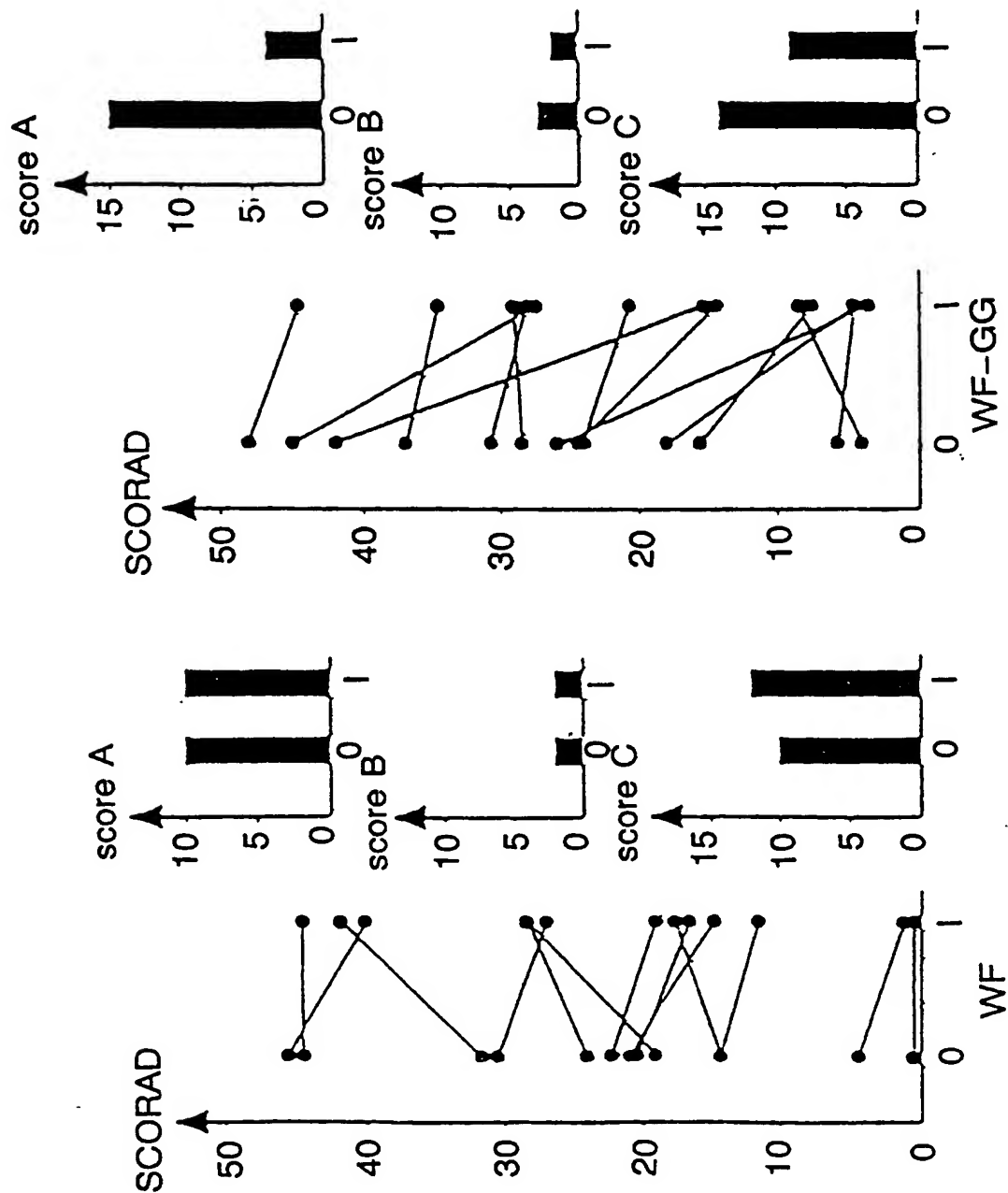


Figure 1C - 1D

3/5

Figure 2



4/5

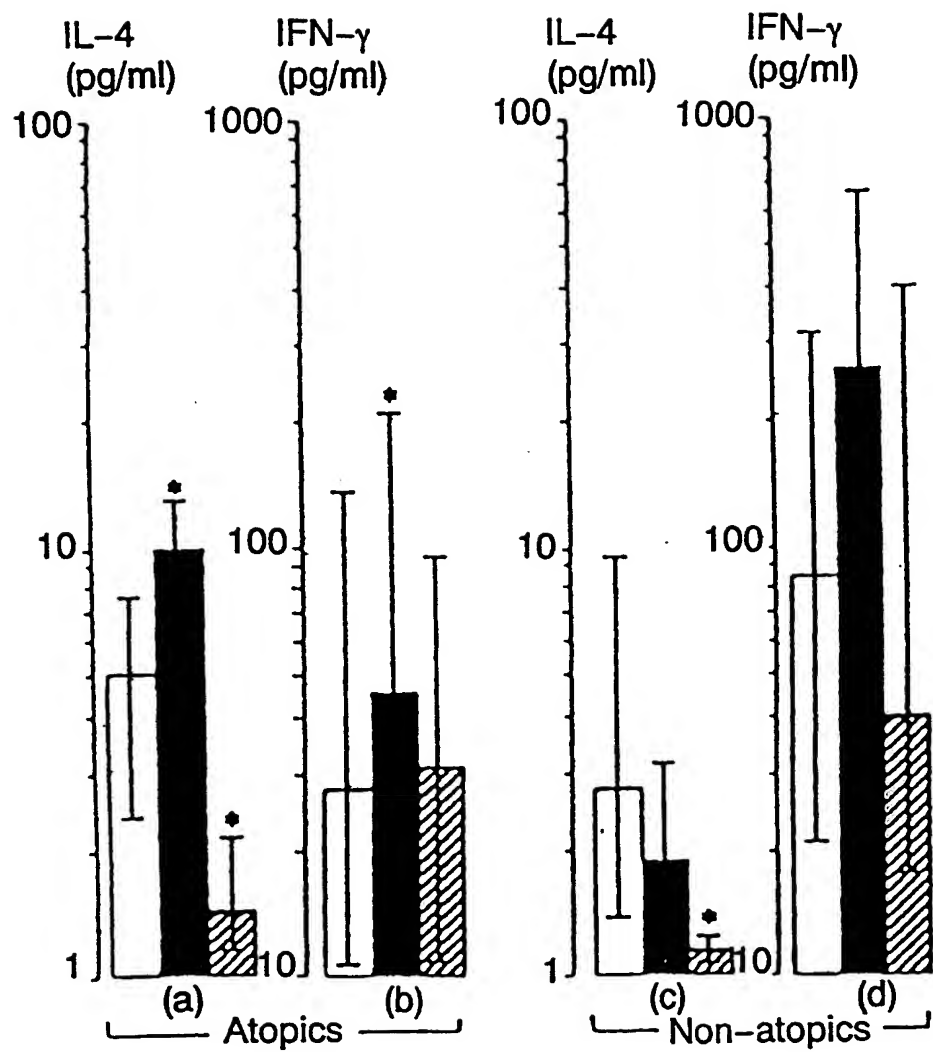


Figure 3

5/5

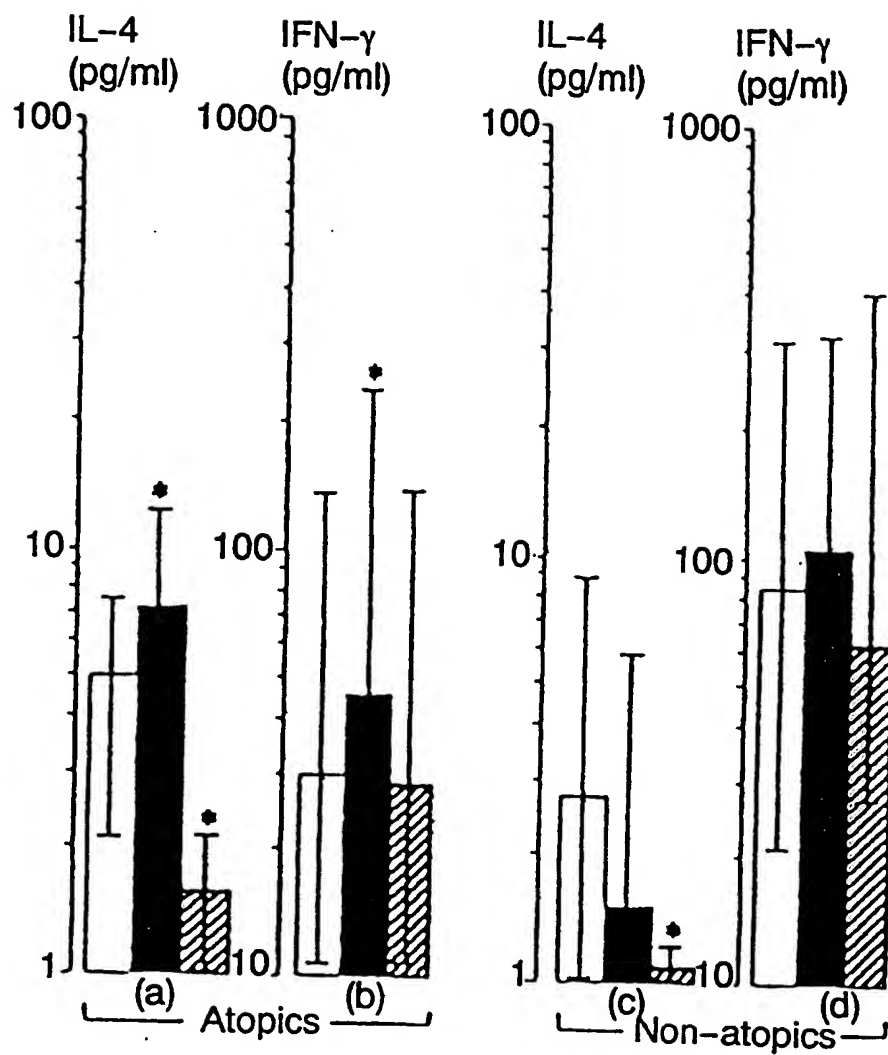


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 96/00350

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/01, A23J 3/34, A23C 9/123, A23C 9/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, A23J, A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, WPIL, FSTA, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 350,WPI, Dialog accession no. 001837412, WPI accession no. 77-58412y/33, Morinaga Milk KK: "Protein decomposition product prepn. - having no bitter taste and no antigen activity"; & JP,A,52079083, 770702, 7733 (Basic) --	1-12
X	DD 281540 A5 (VEB BERLIN-CHEMIE), 15 August 1990 (15.08.90), see the claims --	1-12

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

- Special categories of cited documents:
- *A* document defining the general state of the art which is not considered to be of particular relevance
- *B* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *Z* document member of the same patent family

Date of the actual completion of the international search

13 Sept. 1996

Date of mailing of the international search report

18 -09- 1996

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Palmcrantz
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 96/00350

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 351, WPIL, Dialog accession no. 010305519, WPI accession no. 95-206779/27, Wado Doctors Group KK et al: "Compsn. contg. live enteral bacteria as anti- allergic agent - useful for improving intestinal flora, for treating atopic dermatitis, allergic rhinitis etc."; & WO,A1,9514485, 950601, 9527 (Basic)	11-12
A	--	9-10
A	EP 0629350 A1 (SANDOZ NUTRITION LTD.), 21 December 1994 (21.12.94), page 3, line 1 - line 2, the claims	1-12
A	-- Dialog Information Services, file 155, Medline, Dialog accession no. 08759844, Medline accession no. 94074844, Isolauri E. et al: "Lactobacillus casei strain GG reverses increased intestinal permeability induced by cow milk in suckling rats", Gastroenterology (UNITED STATES) Dec 1993, 105 (6) p1643-50	1-12
	-- -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI96/00350

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 6-12
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 6-12 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition (c.f. PCT Rule 39.1(iv)).
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

31/07/96

International application No.

PCT/FI 96/00350

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
DD-A5-	281540	15/08/90	NONE		

EP-A1-	0629350	21/12/94	AU-A-	6471494	22/12/94
			CA-A-	2125793	17/12/94
			JP-A-	7101873	18/04/95

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.